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**14. ABSTRACT**

Aim 1: Loss of CD44 standard and increased splice variant form CD44v7-10 facilitate prostate cancer (PC) invasion. Mitogen-activated protein kinase (MAPK) pathways and paracrine calcitonin may dysregulate CD44. CD44 total and CD44v7-10 RNA or protein were assessed in androgen-independent PC with known high CD44v7-10 expression and in BPH-1 cells in response to exogenous calcitonin and to inhibitors of protein kinase A, MEK, JNK, or p38 kinase. Inhibition of MEK or p38 but not JNK reduced CD44 RNA in cancer and benign cells. Calcitonin, in calcitonin receptor-positive cells only, caused suppression of CD44 total but increase in variant, the latter apparently dependent on the p38 pathway. See the attached, submitted manuscript. Aim 2: In vivo trials of altering CD44 in mouse xenografts. We spent several months cloning our DNA sequences for CD44 standard overexpression and CD44 variant RNAi out of pTracer and into pAAV-IRES-GFP to use the latter plasmid to establish therapeutic adenoassociated virus (AAV). However, when C4-2 prostate cancer cells were infected, only 30% were GFP+ infected cells, and the low percent of viable cells, due to viral cytopathic effect, prohibited in vitro or in vivo use of AAV. We decided to revert to the pTracer approach with blasticidin cell selection. Matrigel invasion assay demonstrates that non-invaded cells have much higher GFP+ than invaded ones and by western blot analysis, non-invasion correlates with altered CD44 standard and variant.

**15. SUBJECT TERMS**
CD44, cell adhesion, alternate splicing, mitogen-activated protein kinase, calcitonin, pTracer plasmid

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INTRODUCTION:
CD44 is a transmembrane cell adhesion glycoprotein whose alternative splicing produces protein products implicated in tumor development. In Aim 1, we used real-time RT-PCR and western blot analysis to show significant alterations on CD44 transcription, splicing, or both, caused by 1) exogenous calcitonin and 2) inhibition of certain components of the mitogen-activated protein (MAP) kinase pathway. In Aim 2, we had planned to use plasmid vectors to re-express CD44 standard isoform (anti-tumorigenic) or perform RNA interference against CD44 variant (pro-tumorigenic) in prostate cancer cells in mouse xenografts. In preparation for use of adeno-associated virus (AAV) in place of plasmid, which has numerous advantages, we moved our constructs for altering expression into AAV.

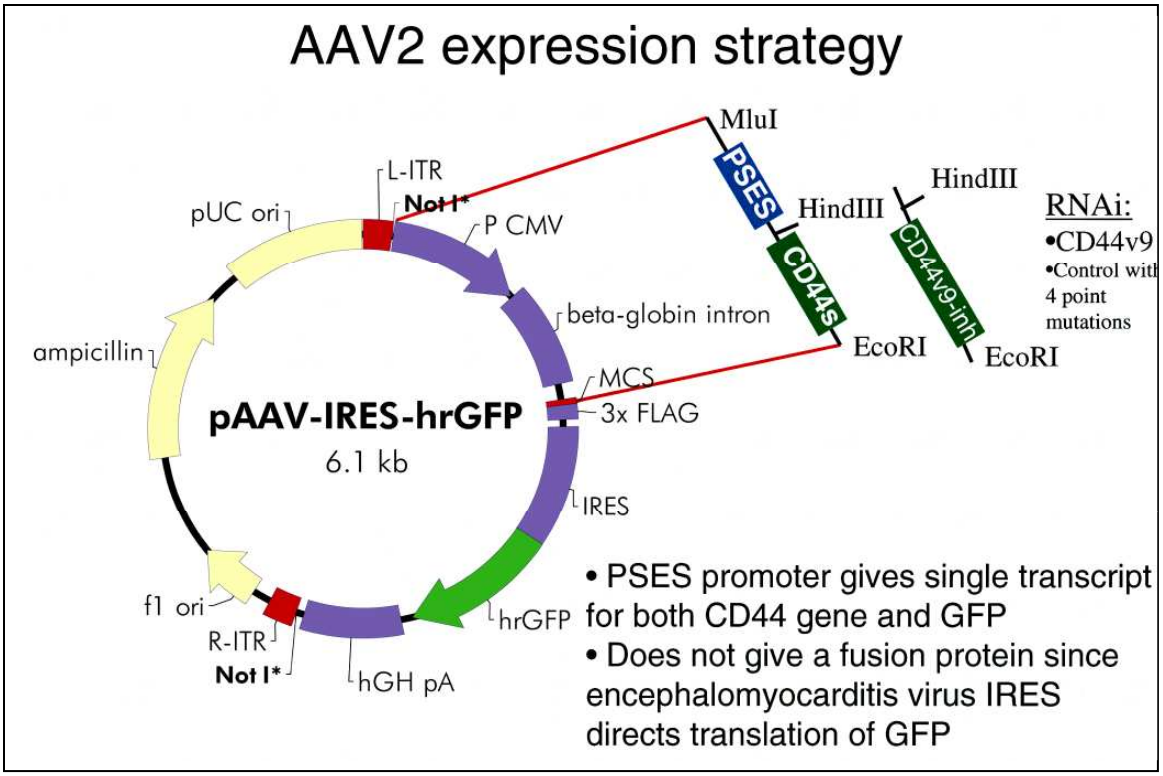
BODY:
Note on Timing and Personnel: My progress was limited because I had recently moved from the Gainesville, Florida, Veterans Affairs Medical Center and University of Florida to the University of Colorado, with a start date at work of March 23, 2007. Although funding became effective April 1, 2007, it took time to get a Research Assistant. Delays were due to the transfer of the award from my old institution (University of Florida) to my new one (University of Colorado Health Science Center); to the physical necessities of the move; and to the need to recruit by advertising; and to administrative start date constraints. Mr. Eric Robbins, MS (Microbiology) was hired July 1, 2007, as *Research Assistant* 3 months after the start of DOD funding.
Using my start-up funds, Dr. Emily Travanty, Ph.D. (Microbiology) was hired as a *Fellow* September 1, 2007. Dr. Travanty had completed a 3-year fellowship in virology and remained with our laboratory for four months, at which time she left to take a Research Scientist position at another institution. In April, 2008, Dr. Kui Yang, Ph.D., was recruited as a *Fellow.*

Aim 1: Mitogenic Pathway Effects on Splicing: Mr. Robbins' duties from July 1—December 1 were primarily concerned with Aim 1's first sub-Aim involving MAP kinase component involvement in CD44 splicing (1).
Our CD44 variant primer/probe set (from Florida) was suboptimal because the primers were too short and had a suboptimal G+C percent. The weeks from August to mid-September were taken up re-designing primer/probe sets for TaqMan for CD44 total (standard plus CD44v) and for CD44v7-10; with several experiments optimizing annealing temperatures and concentrations of probe and primer for the CD44s, CD44v, and the 18S ribosomal RNA control; and gaining proficiency making non-degraded RNA preparations. This involved some regular RT-PCR. RNA was isolated from treated or mock-treated cells in each experiment and examined by electrophoresis and optical densitometry.
We broadened this Aim to include not only testing of MEK and JNK inhibitors, but also p38 inhibitor, listed in the Alternatives section of this Aim. Further, involvement of the enzyme upstream to these three pathways, protein kinase A (PKA), was tested using its specific inhibitor H89.
Also, the effect of exogenous or endogenous calcitonin (CT) and its receptor (CTR) were investigated. The rationale for this is my decade-long collaboration with Dr. Girish Shah

(2-6) (Univ. of Louisiana) and his compelling demonstration of functional roles of CT and CTR in prostate cancer growth and invasion (7-17). As depicted in Fig. 7 of my proposal, we propose an action of CT-CTR axis on PKA. The response of CD44 total (mostly CD44s) and CD44v7-10 to exogenous CT was elucidated.
Endogenous CT was studied by increasing the number of cell lines or variants tested to include more than just PC-3, the derived $G_s\alpha$-QL, and LNCaP. Note that PC-3 cells are CT+ CTR-, whereas PC-3M cell line is CT+ CTR+ (17). The PC-3M variants with knockdown of calcitonin (CT-) or of its receptor (CTR-) were obtained from Dr. Shah and also tested.
From mRNA preparations, a series of 37 Real Time (TaqMan) RT-PCR runs were carried out. From protein preparations, numerous Western blot analyses were done.
A manuscript based on this work was submitted to *Cancer Letters* in Dec. 2007, revised and re-submitted to *International Journal of Cancer* in April, 2008 (**Appendix**).
Metabolic labeling of proteins: This sub-Aim is a metabolic study based on answering the question, why did overexpression of CD44s cause an inhibition of CD44v. It would involve radio-labeling the endogenous CD44v. As an alternative, we have developed luciferase vectors for CD44 promoter (altered transcription) and CD44v splicing (**Appendix 1**). When stable PC-3 cells are developed expressing the luciferase vector for CD44v splicing, these can be used to assess a direct effect of CD44s overexpression on CD44v splicing.

Aim 2: This Aim was mainly pursued by Dr. Travanty in her 4 months (Sept.-Dec.) and was assumed by Mr. Robbins (Jan. 2008).
We have not worked with mice yet. We decided to try to improve our approach over that described in the proposal and to perform a series of *in vitro* studies in prostate cancer. The improvements required a shift in strategy from a plasmid delivery system to an adeno-associated virus 2 (AAV2) approach.
When I was still at Univ. of Florida in the fall of 2006, I obtained from Dr. Arun Srivastava, a virologist, the plasmid pPSES/TACI/JQ-1. PSES stands for Prostate-Specific Enhancing Sequence, and has promoter-like activity but only in cells that are PSA-positive (18-20). The CD44 overexpression construct was cloned in but *without* GFP gene to mark transfected cells green. The CD44 overexpression sequence was cloned out of pTopo plasmid with the ITR parts. In vitro invasion experiments looked promising.
After moving to Colorado, I received some guidance from Dr. Jerome Schaack in the Health Science Center's Microbiology department concerning how to get GFP into the plasmids of interest. We decided to start over and clone first our PSES-CD44 standard overexpression construct (with and without FLAG tag) out of plasmid vector pTracer and into pAAV-IRES-GFP (Stratagene). Subsequently, we would excise the CD44 standard and replace it with CD44 variant RNAi construct (with or without 4 conservative mutations, **Fig. 1** ):

**Fig. 1**




This task was arduous, took months, and was fraught with frequent setbacks. In the end, after about five months of work to convert to a viral approach of gene expression, we reverted to using plasmid to overexpress or cause RNAi of the genes of interest. Since much time and effort were expended in the viral approach, we have detailed our work in this report (**Appendix 2**). In retrospect, studies on cell viability after viral infection should have been carried out earlier, building AAV using empty vector plasmid.

All the above work was suspended when we discovered in March, 2008, that infection of the C4-2 cells with the viral constructs was too cytotoxic to be workable. The percent of green GFP+ cells did not approach 100% and FACS cell sorting was required. Cells were either dead or dying at the time of FACS cell sorting so that no viable cells could be recovered. Thus, we reverted to a non-viral approach using PC-3 cells and stable pTracer plasmid for CD44 standard overexpression, or CD44v RNAi.
To confirm that the tumorigenicity of the PC-3 cells is altered before use in mice, we transfected PC-3 prostate cells with Empty Vector, CD44v9 RNAi, and CD44s overexpression and tested them with a series of *in vitro* assays that is in progress

1. Matrigel Invasion Assay as per Omara-Opyene et al. (4) was done, followed by western blot analysis of the invaded versus non-invaded cells, compared with untreated PC-3 cells (**Fig. 2**).

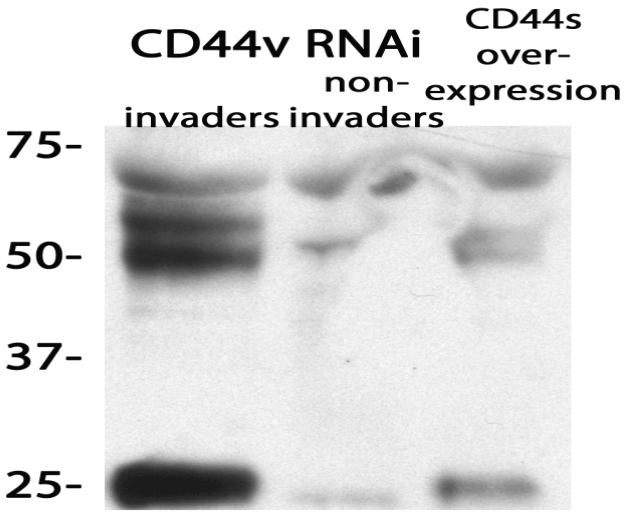
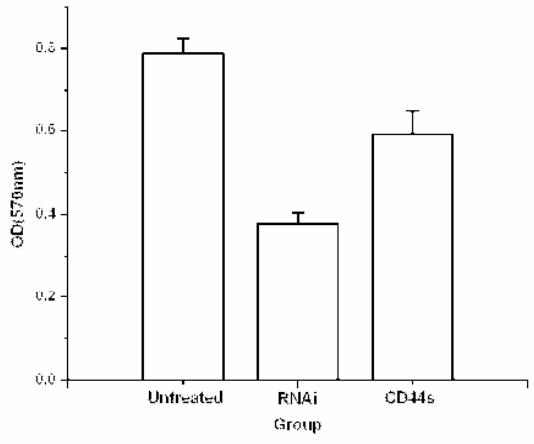



**Fig. 2** Western blot analysis of C44v9 in PC-3 cells. **Lanes 1-2**: with RNAi, the cells that invaded Matrigel after 36 h (**Lane 1**) have greater expression of the 66 kD bands as well as the 25 kD cleavage product, compared with non-invaded cells (**Lane 2**) after 36 h. **Lane 3**: Cells with enforced CD44s overexpression also show relatively less CD44v. (We are aware of the technical flaws here and plan to repeat this blot with protein preparations. Also, the bands for untreated PC-3 cells were technically inadequate so are not shown here.)

2. MTT (Sigma-Aldrich or ATCC) colorimetric survival assay for proliferation as in Thomas et al. (14). PC-3 cells were treated for 72 hours with lipofection agent and DNA to cause RNAi or CD44s overexpression. Percent of GFP+ cells were counted. Cells were allowed 12 hours to recover from the lipofection. Then, RNAi treated cells and no-treatment PC-3 cells were partially harvested from respective flasks. A third sample was obtained by putting blasticidin in the flask of RNA-transfected cells to kill non-transfected cells for 48 hours. After large numbers of cells detached the floaters were removed. The attached cells were given 24 hours in medium without blasticidin to recover, and the percent of counted cells that were GFP+ were assessed.
Triplicate wells of 80,000 cells/well were plated out on a 96-well plate 24 h prior to the experiment. Cells were incubated 2 h at 37 C followed by cell lysis with 1 mL lysis buffer with 20% SDS for 6 h. Cell lysates examined for optical absorbance at 595 nm. Viable cells = optical absorbance treated/optical absorbance control. ANOVA with 2-sided test was performed.

**Fig. 3** MTT assays. RNAi against CD44 v7-10 brought about 50% decrease in proliferation, while overexpression of CD44s caused a 25% decrease. Error bars are ±S.D.




The cells treated with RNAi were observed by microscopy in the wells prior to the experiment. They had 5% GFP+ green cells under immunofluorescence. Cells with CD44s overexpression had 4.1% GFP+ green cells. The RNAi proved more strongly inhibitory to proliferation than the CD44s overexpression (**Fig. 3**). The phenomenon of functional effect being out of proportion to the percent of GFP+ cells suggests that more

cells are transfected than are showing green. We published the same phenomenon in Matrigel invasion assays in 2004: the percent inhibition in Matrigel invasion exceeded the percent of GFP+ cells (4). See Fig. 3.
3. Soft agar assay for colony formation as per Chien et al. (8). In progress.
4. Spheroid formation and migration assay on vitronectin as per Chien et al. (8).
5. Possibly assay cells for differential response to stress (chemo/radiation) by exposing cells to DNA damaging agent Mitomycin C (Fisher Sci.) and analyze growth at daily intervals from days 1-5 as in Zhang et al. (21).

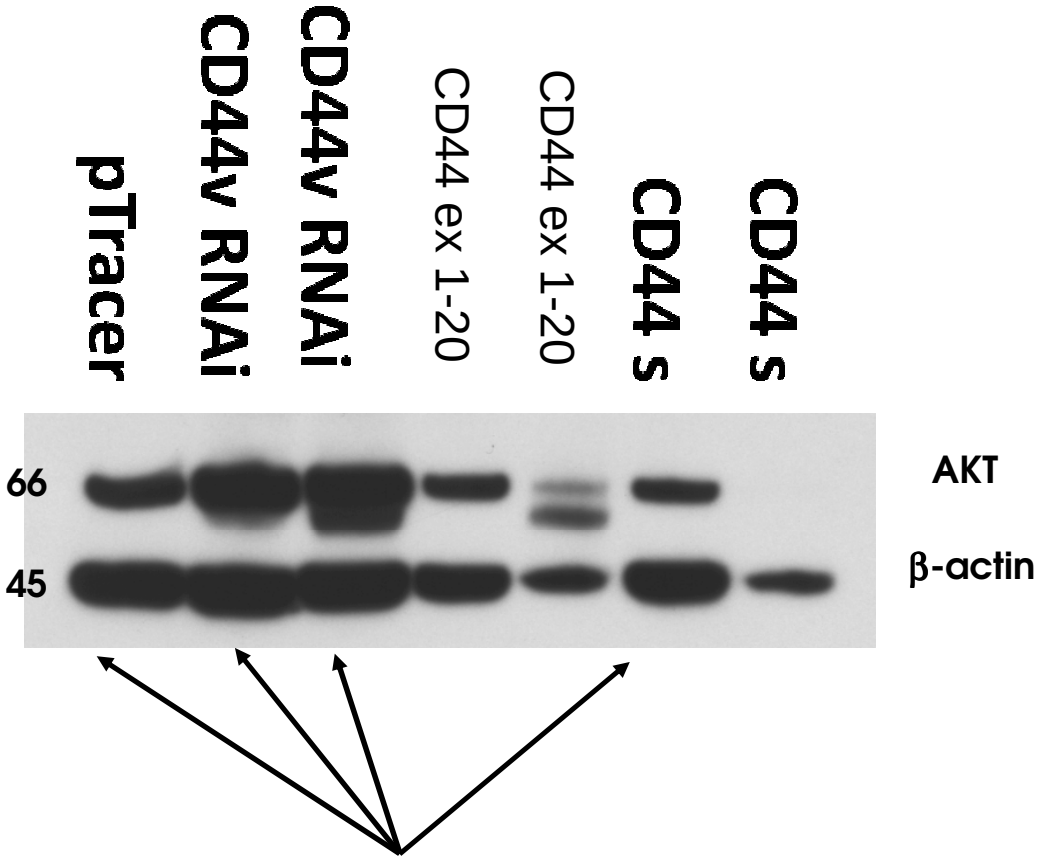
After these tests, either pretreated PC-3 cells will be grown subcutaneously in nude mice or untreated cells nwill be first grown and then treated by subcutaneous injection for the gene of interest. The same numbers and groups of mice as originally stated in the proposal will be used.

KEY RESEARCH ACCOMPLISHMENTS:
1. Completion of sub-Aim 1 under Aim 1; submission of a revised manuscript (see Appendix).
2. Preparation of luciferase constructs for CD44 promoter activation (using a construct from Dr. Jim Lambert of our Department) and for CD44 variant splicing. These have great potential to supplement our work to come. In Aim 2, when stably transfected cells are to be grown in mice, the extent of CD44 alteration can be verified using the luciferase assay instead of more complicated assays such as western blot and qRT-PCR. Thus, the constructs can confirm CD44 total or variant alteration. For those using cells transfected by intratumoral injection, the constructs can be co-transfected to examine extent of CD44 alteration at harvest. Since we cannot be certain GFP is indicative of the proportion of transfected cells, this will be a valuable adjunct.

In February, the CD44 variant splicing pET construct and empty pET vector were obtained from Dr. Harald Koenig in Germany. They had a CD44v5 splicing construct for use with lymphocytes (22), but we removed it and replaced it with CD44 v7-10 in the multiple cloning site. These constructs were prepared successfully and provide an additional, easily used tool to test mechanistic influences on CD44 transcription and alternate splicing. The 8 weeks of work done on this project are detailed in **Appendix 2**.

3. Because of the importance the ezrin-moesin-merlin (ERM) complex in mediating the cytoplasmic signaling of CD44 (23-24), we obtained a plasmid for Merlin overexpression (Dr. D.H. Gutmann, Washington University) and grew larger amounts. Future experiments may test its effect on CD44 variant expression in prostate cancer.
4. Some of our colleagues have an interest in the Akt pathway in prostate cancer. Furthermore, the AKT pathway interacts with the MAP kinase pathway in cancer cell growth (25). By Western blot analysis, total Akt and phospho-Akt were studied in protein preparations from PC-3 cell whose expression of CD44 was altered previously (5,6) using pTracer plasmids. We found that total AKT levels are increased in cells exposed to the RNAi construct (**Fig. 4**). This may be a non-specific stress response due to activation of RNAi pathway. For cells with overexpression constructs of CD44 exons 1-20 or CD44 standard, there was no comparable AKT increase observed.

**Fig. 4** Western blot analysis for total AKT




5. Because of the interest of some of our colleagues in the Univ. of Colorado Pathology Department's Prostate Cancer Research Laboratory (26) in the tumor suppressive effects of vitamin D (27, 28), we tested the effect of vitamin D and an analog on CD44 expression in $G_s\alpha$-QL and ALVA-3 (a PC-3 derivative) cells. This work was done in 9/07-11/07. Effects of vitamin D or its butyl ester (BE) analog (1α,3ß,24$(OH)_3$-22-ene-24-cyclopropyl-25-n-butyl-ester-vitamin $D_3$) at $10^{-7}$ or $10^{-8}$ M doses, or of the vitamin D receptor antagonist ZK159222 were tested for 24 hr. TaqMan assays for CD44s or CD44v levels gave some hints of a change but results were not consistent. However, now that we have *luciferase promoter construct and splicing construct*, we will have another modality to test whether vitamin D or its analog cause CD44 transcription or splicing changes in prostate cancer cells, and we plan to use it.

6. Because of the interest at Univ. of Colorado in the nutritional chemoprevention of prostate cancer by the anti-growth compound Silibinin, we tested the effect of this compound on CD44 total and CD44v7-10 RNA by TaqMan, and found an inhibitory effect on CD44 total and CD44 variant (**Fig. 5**).

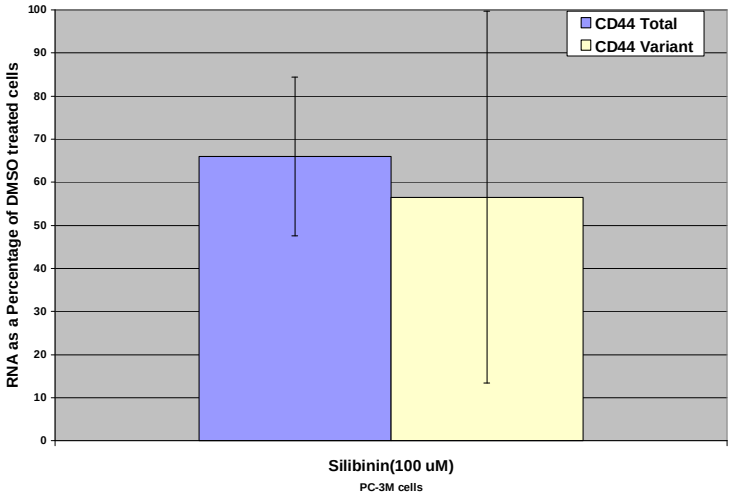



**Fig. 5**. qRT-PCR shows less CD44s and CD44v in Silibinin-treated cells.

This has led to a collaboration with Dr. Thomas Flaig (Urologic Oncology) who has access to prostatectomy tissues from patients treated with Silibinin. We are obtaining IRB protocol approval to perform immunostaining of the prostate tissues for CD44v9, to demonstrate Silibinin's likely decrease in this marker of tumor aggressiveness. Additionally, we plan to use the *luciferase promoter construct and splicing construct* to test further the effects of Silibinin.

7. Early growth response-1 (Egr-1) gene cDNA was made from leukemia cells using specific primers (29). The PI's interest in this prostate cancer marker extends back a decade (30). EGR-1 is a transcription factor induced by stress or injury, mitogens and it is possible it may mediate influence of growth factors on CD44 splicing (31). A future project may be to overexpress Egr-1 in prostate cancer cells to uncover a mechanism for alternate splicing.
8. We obtained anti-CD44 v9 producing hybridoma cells from the ATCC and grew these cells to produce mouse monoclonal antibody-containing supernatant. These supernatant stocks replace less-effective 2-3 year-old stocks and will be used for Western immunoblots, and for the immunoprecipitation in Aim 1, second sub-Aim. They will also be used for immunostaining mouse tumor tissue in Aim 2.

REPORTABLE OUTCOMES:
Manuscripts: One manuscript has been submitted. The PDF is in the Appendix.
Presentations: Our data have been repeatedly shared and discussed at the weekly Prostate Cancer Research Laboratory meetings, Univ. of Colorado Health Science Center.
Cell lines: We are establishing stably pTracer plasmid expressing PC-3 prostate cancer cell lines for CD44s overexpression, CD44 variant RNAi, and as controls, CD44 variant RNAi with 4 conservative mutations, and empty vector. All will be used for Aim 2.

CONCLUSIONS: The past year has seen progress in MAP kinase and calcitonin studies with regard to CD44 transcription and alternate splicing in prostate cancer cells (Aim 1), resulting in a submitted manuscript. We also have made AAV2 virally-transfected C4-2 prostate cancer cells, but we reverted to the plasmid approach using PC-3 cells. We now have performed or are performing several *in vitro* invasion and proliferation assays that our laboratory had not done previously. These assays were necessary as a prelude to *in*

*vivo* gene therapy (Aim 2). We have IACUC approval and will soon begin the somewhat less technically complex task of *in vivo* growth of tumor in nude mice.

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**APPENDICES:** See also our manuscript, *MAP Kinase Pathways and Calcitonin Influence Alternate CD44 Isoform Expression in Prostate Cancer*.

## APPENDIX 1: Preparation of Luciferase Expression Vector Constructs, Completed by April 22, 2008

CD44 Promoter construct

2/20-Diluted CD44 promoter HindIII-XhoI primers, set up PCR to amplify promoter. PCR failed.
2/22-Ran PCR using Dr. Jim Lambert's Epicentre buffer kit and our homemade pfu. Two PCRs succeeded, using buffers K and J. Cut out band.
2/25-Set up PCR to re-amplify this band using pfu buffer on hand as on 2/22. PCR failed.
2/27-attempted to re-amplify PCR band from 2/22 using buffers K and J. Fragment amplified, but less than before.
2/28-Ran original 2/22 PCR on gel, gel purified, ran Taq A overhang addition reaction without primers, ran reaction to clone fragment into pCR2.1-TOPO plasmid. Transformed cells with this plasmid.
2-/29-No colonies on plates. Ran out band to ensure it was not degraded. Band faint but present. Ran PCR to re-amplify promoter. PCR failed
3/5-Ran PCR to amplify promoter using Epicentre buffer kit. PCR yielded one faint band. Extracted band
3/6-Tried to amplify above band. PCR failed.
3/7- Tried PCR again with Epicentre enzyme mix as well as buffers. Worked much better.
3/10-Extracted bands from above PCR. Ran Taq A addition reaction and pCR2.1 TOPO inclusion reaction, transformed cells.
3/14-Received new primers, performed PCR to amplify promoter using homemade pfu (ran out of Epicentre enzyme). PCR failed. Perhaps too little enzyme used.
3/17-Ran PCR to amplify promoter again with more enzyme in buffers D and J (have worked before). PCR failed.
3/20-Ran PCR again with all buffers. PCR failed. Concluded the primers constructed improperly based on sequences from splicing construct (Not why PCR hasn't been working, but would introduce SacI site instead of XhoI site). Reordered primers.
3/26-Attempted PCR with new primers and lowering annealing temp to 50 degrees. Failed. Since kit worked so much better with Epicentre enzyme, bought new kit.
3/31-New kit arrived. Tried PCR to amplify fragment. Worked.
4/1-Extracted PCR band, set up Taq A addition reaction and pCR2.1-TOPO inclusion reaction. Transformed cells.
4/2-Picked colonies, grew up in broth.
4/3-Purified plasmid. Sent for sequencing
4/9-Sequences came back. Sequences good, but SacI site instead of XhoI site on the end. Ran PCR from these plasmids using old primers.
4/10-Extracted PCR fragment from gel. Ran Taq A addition reaction and pCR2.1-TOPO inclusion reaction. Transformed cells.
4/14-Picked colonies and grew up in broth.

4/15-Purified plasmid. Conducted XhoI digest to ensure correct XhoI sites. Looked good. Sent to sequencing.
4/17-Sequencing good. Obtained PXP2 luciferase plasmid from Dr. Steve Nordeen. Cut pCR2.1-TOPO-CD44prom and PXP2 with HindIII and XhoI. Ran on gel and extracted bands.
4/20-Set up ligation.
4/21-Transformed cells.

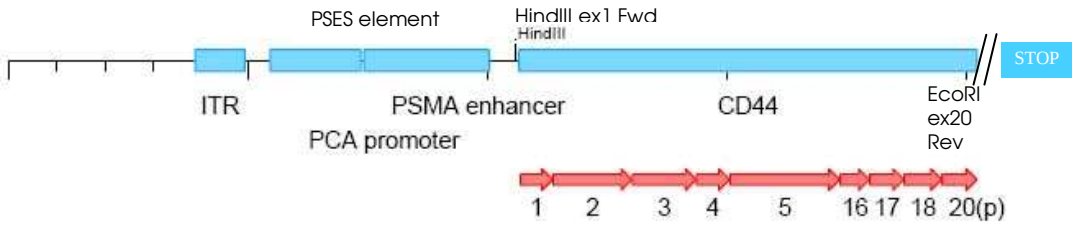
CD44 Alternate splicing construct
2/26-Transformed E. coli with pET EB Cut Luc from Koenig in Germany (22) to make splicing construct plasmids, grew up plasmids
2/29-Diluted CD44v10 SacI-NotI primers, set up PCR to amplify CD44v10 using Epicentre buffer kit. PCR succeeded for all buffers.
3/3-Ran 2/29 PCR on gel, extracted bands. Ran Taq A addition reaction and reaction to clone fragment into pCR2.1-TOPO. Transformed cells.
3/4-No colonies on plates. Purchased new TOPO-TA cloning kit. Performed Taq A addition reactions with and without primers. Performed pCR2.1-TOPO cloning reaction. Transformed cells
3/5-Primer Taq A addition reaction yielded many more colonies than without primers. Picked colonies to grow up.
3/6-Purified plasmid from above colonies. Sent for sequencing.
3/11-Got sequences back. Included SacI site in primers in the wrong orientation-actually an XhoI site. Reordered primers with SacI site in the proper orientation.
3/14-Received new primers, performed PCR to amplify promoter. PCR failed. Perhaps too little enzyme used.
3/17-Ran PCR to amplify v10 again with more enzyme. PCR succeeded.
3/18-Extracted v10 bands, ran Taq A addition reaction and pCR2.1-TOPO inclusion reaction. Transformed cells.
3/19-Picked colonies, grew up.
3/20-Purified plasmid from colonies. Ran SacI digest on plasmid for 2 hours. Followed up with NotI digest. NotI cut when used separately, but no insert for sequential digest. Concluded SacI did not have long enough to cut. Also submitted plasmids for sequencing.
3/21-Ran SacI digest 12 hours, ran on gel. Cut after 12 hours but not 3.
3/24-Sequences came back good.
3/25-Ran Not I digests of plasmids followed by SacI digest of plasmids. NotI cut. SacI did not produce inserts after 2 hours, suggesting that it did not cut. Left SacI digest overnight at 37 degrees.
3/26-No insert after overnight SacI digest. Bought new SacI from a different vendor (Roche). Attempted digest again.
3/27-Digest with new SacI worked. Set up NotI digest. Ligated NotI digest into pET cut EB Luc.
3/28-Transformed cells with ligation
4/29-Ligation plates covered in bacterial lawn. Thinking Ampicillin might be bad, tried different plates.
4/2-Again plates covered in lawn. Tried digests again

4/3-Bands appear but are too small/faint to extract. Decided to repeat SacI digest (which should yield a larger band) and digest this band with NotI.
4/4-Digested pCR 2.1-TOPO CD44v10 and pET cut EB luc with SacI overnight.
4/7-Extracted band from 4/4 digests. Digested with NotI. Attempted to ligate insert into vector
4/8-Transformed cells.
4/9-Again too many colonies. Dr. Lambert says the vector is not cutting completely and to use alkaline phosphatase to prevent once cut vector from ligating back together. Attempted this, and ran out on gel. No band-vector too dilute. Ordered thermosensitive alkaline phosphatase (TSAP) from Promega- can be heat killed and can work in restriction buffers, meaning the DNA does not have to be purified and therefore diluted after every step.
4/15-TSAP arrived. Conducted overnight SacI digest of vector.
4/16-Conducted NotI digest simultaneously with TSAP treatment. Heat killed enzymes. Set up ligation.
4/21-Grew up colonies from ligation.
4/22-Purified plasmid and digested with NdeI to confirm insert

## APPENDIX 2: Preparation of Plasmids with Expression Driven by PSES Enhancer, for Use in AAV Viral Approach, completed February 2008

The potential advantages of viral delivery over plasmid delivery of DNA that prompted us to work on the viral approach were as follows:

1) We were concerned about the safety of altering CD44 expression systemically. Even if CD44-altering plasmid or viral therapy were injected into the prostate, it could alter CD44 expression of healthy kidney, gastrointestinal, bladder and other organs in untoward ways. Thus, part of Dr. Travanty's work was to put the CD44-altering DNA under transcriptional control of the PSES promoter, which restricts expression to PSA-producing cells only:

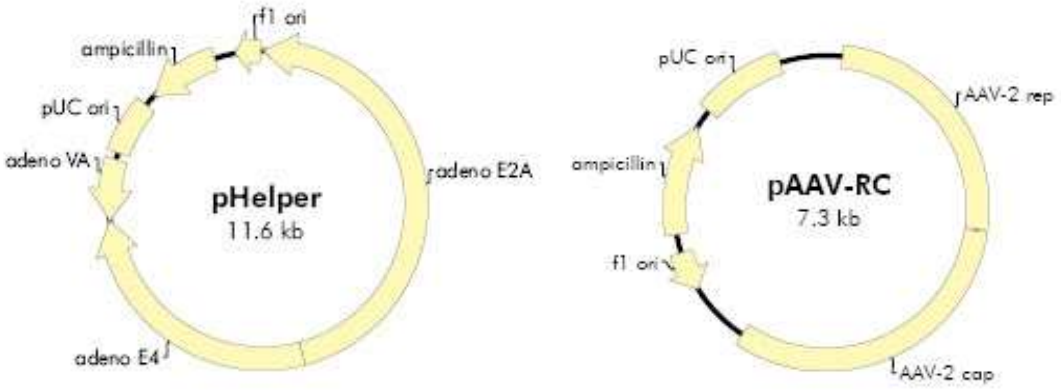



Note that PSA expression by cancer in human prostatectomy tissue is usually less than in benign prostate, but it is still fairly strong. Among cancer cell lines, certain lines such as PC-3 and DU-145 are PSA-negative. Thus for both in vitro and in vivo work, use of a PSA-positive cell line was necessary and we chose C4-2. C4-2 cells are an LNCaP derivative but differ because of faster growth and unlike LNCaP have metastatic potential when introduced intracardially, or as xenografts or orthotopic prostate grafts.

2) Preliminary data generated in Florida from 2005-06 showed us that only a minority (far less than 50%, at best) of prostate cancer cells could ever be transfected by pTracer constructs, as demonstrated by the proportion of cells expressing the associated GFP green under fluorescent microscopy. The same was true for xenograft mouse tissue harvested from mice: only certain cells were green using direct visualization of GFP, and the GFP distribution was patchy. **AAV2 gets into cells very efficiently** so we expected a much higher percent of green cells for human gene therapy, which is the ultimate goal of our research.
3) It is well-known that plasmid gene therapy, while often effective, *is transient*. This was certainly true in our hands. Cell lines lost their GFP as well as their altered CD44 expression after more than a few passages. In vivo, it was necessary to perform intratumoral injections of plasmid 5 times per week for the entire treatment period of about 5 weeks in order to get a halt or shrinkage in tumor growth. Almost daily intratumoral injection in men with prostate cancer (in either the prostate or metastatic site such as lumbar spine) would be impractical. **The ability of AAV2 to integrate into human cells stabilizes the change in gene expression.** For human gene therapy, then, the desired stable alteration of CD44 expression is achievable only with a viral vector.
4) AAV2, unlike adenovirus, lacks an undesirable side effect profile.

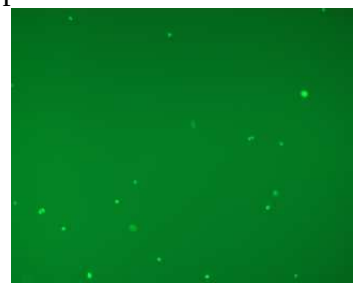
Progress from 10/07 to 1/08 was as follows:
10-11 We had first built plasmid pPSES-CD44 (overexpression construct) from a TACI plasmid containing the PSES promoter (gift of Dr. Arun Srivastava, U of Florida). Our first efforts were to move the PSES-CD44 construct. An attempt was made to place PSES-CD44 into a pTracer as a cloning vector.
10-15 The expected 1.1 kb CD44 gene failed to drop out using XbaI and HindIII.
10-16 Attempted digestion was incomplete.
10-18 New cloning primers had to be ordered because of concerns over the fragment's large size.
10-23 Sequencing disclosed that the tail end of CD44, or exons 19-20, was not present in the attempt to clone the full length PSES-CD44.
10-24 Thus, we examined the presence of the cytoplasmic tail in RNA preparations from 8 clinical benign and tumor prostate samples.
10-25 CD44s with exons 1-5 and 16-20 is predicted to be 2.7 kb. Sequencing using special primers disclosed, in all samples, a 979 bp product that corresponded to exons 1-5 and 16-18 plus part of 20. There was a much rarer 2.0 kb product containing the very long exon 19 but none of exon 20. Thus, **PC CD44s does not normally contain all of both exons 19 and 20**.
10-26 RNA preparations from leukemia cells were tried in order to isolate the CD44 with the full-length tail.
10-30 Work was begun on pTracer-CD44v9 RNAi clone #45, that we used before (37,38). Planning for the need for a control fragment for RNAi, we designed and ordered mutagenesis primers with 4 conservative mutations as per our plans in the grant proposal.
11-2 RNA preparations from leukemia cells disclosed CD44 without exon 19 and most of 20; breast cancer MCF-7 cells were tried.
11-9 Gel extraction problems required purchase of a new kit.

11-12 Sequencing revealed that there was no exon 19 and most of 20 in breast cancer, either. Notably, because the literature confirms that CD44s is generally missing exons 19 and most of 20 (21,22), and exon 20 is the one required for hyaluronan binding (21), we concluded **it was irrelevant that these portions of exons were missing** from the CD44s overexpression fragment in PC for this purpose.
11-13 Double digests of pAAV-IRES-GFP-PSES-CD44 with EcoRI and Xba I failed, thus sequential digests were deemed necessary.
11-14 Sequential digests worked; we transformed TOP10 competent E. coli. The appearance of colonies on the empty vector plate suggested the Ampicillin was ineffective.
11-15 The plating was repeated, and although there were still colonies with vector-only, the overexpression clone #2 was found to be **good** and was sequenced.
11-19 After a meeting with protein chemistry expert Dr. David Orlicky of our Department, we set a goal of adding a FLAG tag to the CD44 overexpression fragment and produced other CD44 clones without the tag.
11-21 All colonies picked of CD44-FLAG did not contain the insert but were empty vector. Vector had to be re-digested.
11-29 CD44 overexpression—clones with no tag looked good.
12-3 CD44-FLAG clones again lacked the insert.
12-7 In order to move ahead with viral preparation, we transfected AAV-293 cells with **empty vector** (EV) pAAV-IRES-GFP, along with plasmids pHelper and pRC, and harvested viral **stock**.




12-10 Moving ahead with siRNA plasmids against CD44v9 (and control for RNAi), we tried triple ligations. This complex process failed.
12-11 We went back to the second vial of CD44 overexpression-FLAG PCR product.
12-14 Miniprep cultures for CD44 overexpression-FLAG vector were made.
12-18 There were problems with genomic DNA carry-over from the plasmid preparations, so we changed from an alkaline lysis preparation method to a QIAgen miniprep kit.
12-19 DNA of CD44 overexpression-FLAG was sequenced. Problems were still evident with the original CD44 overexpression-FLAG vector.
1-7-08 Triple ligation of CD44 overexpression-FLAG failed again, yielding numerous colonies in the control empty vector. Two-step cloning was substituted.
1-8 EcoRI enzyme was faulty, and unable to digest vector completely.
1-10 The CD44-FLAG overexpression constructs were confirmed as **good**.

1-14 The ampicillin on the plates for the RNAi constructs was faulty.
1-22 The AAV-293 cells were transfected with CD44-FLAG for **overexpression** and viral **stock** was harvested.
1-22 New ampicillin was obtained and plates prepared.
1-26 For the RNAi (siRNA and control) DNA concentrations were low but an attempt was made to transform the ligation reactions into E. coli.
1-30 This attempt failed.
2-2 The RNAi control vector prepared was **good**. The siRNA vector, however, was not completely cut.
2-3 to 2-7 E. coli were re-transformed; the siRNA appeared to have worked but there were too many colonies in the empty vector plate. In the second attempt, digestion with XbaI and EcoRI gave two bands, indicating incomplete digestion. Lengthening digestion time from 1 hour to 3 and purchase of new enzymes remedied this problem.
2-10-08 C4-2 cells were plated in 12-well plates and subjected to varying dilutions of CD44 overexpression viral stock or empty vector viral stock.



2-15-08 Using overexpresion AAV stock, 10% GFP-positive C4-2 cells were obtained at 72 hours (Figure) providing proof of principle. Subsequent TaqMan analysis did not show differential RNA effects from the overexpression. Using empty vector AAV stock, 18% GFP-positive cells were obtained. To obtain 100% green cell populations, cells were sorted by FACS analysis, yielding 1000 GFP+ cells, which were grown in one well of a 6-well plate.
2-17--27-08 With help from a mentor (Dr. Steve Nordeen), we set controls including ligating the empty vector to itself to explain why we so frequently have had no colonies on our culture plates. We re-cloned the CD44 RNAi starting with EcoRI digestion, ran the blunted vectors on gel, and DNA was barely visible. Thus, we switched to a “dirty ligation” with no gel purification performed subsequent digestion and ligation steps.

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# MAP KINASE PATHWAYS AND CALCITONIN INFLUENCE CD44 ALTERNATE ISOFORM EXPRESSION IN PROSTATE CANCER CELLS 4/21/08

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**Abstract**

Two mechanisms implicated in the dysregulated expression and splicing of cell adhesion marker CD44 in cancer are mitogen-activated protein kinase pathways and paracrine calcitonin. In androgen-independent prostate cancer (PC) with known high CD44v7-10 expression, CD44 total and CD44v7-10 RNA or protein were assessed in response to exogenous calcitonin and to inhibitors of protein kinase A, MEK, JNK, or p38 kinase. Benign cells and calcitonin receptor-negative PC cells were also tested. Inhibition of MEK or p38 but not JNK reduced CD44 total RNA in cancer and benign cells. In calcitonin receptor-positive cells only, calcitonin increased CD44 variant RNA and protein by 3 h and persisting to 48 h, apparently dependent on an uninhibited p38 pathway. Thus, the MEK pathway facilitates CD44 transcription, while calcitonin, acting through the p38 pathway, facilitates variant splicing.

*Key words:* prostatic neoplasms; CD44; MAP kinase; MEK; calcitonin; alternate splicing; p38

## Introduction

CD44, a transmembrane glycoprotein, is the product of a gene that can undergo extensive alternate splicing. The standard (**CD44s**) isoform is ubiquitous but tissue-specific isoforms may include an assortment of 10 variant (**v**) exons (**CD44v**). CD44 facilitates multiple cellular functions. CD44 enables cell-cell and cell-matrix adhesion--primarily to its main ligand hyaluronan, and links the cell membrane to the actin cytoskeleton, modulating motility. CD44 is universally dysregulated in human cancer, and this imbalance of isoforms allows tumor growth and invasion.[1-8] CD44v are expressed in prostatic secrectory cells while CD44s is found in the whole epithelium. About 30% of cases of prostate cancer (PC) undergo a transition from quiescent to aggressive. Altered CD44 and other adhesion molecules permit this transition in which tumor cells detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels.

By isolating RNA from clinical PC specimens, we discovered that the major variant isoform expressed in PC is CD44v7-10. This PC signature was consistently present in both primary and metastatic PC.[1-3] Interference against this CD44v caused a 69% reduction in invasion index compared to untreated control cells.[3] Moreover, PC loses the splicing ability to produce the CD44s expressed in benign prostate.[3,9,10] CD44 must oligomerize to bind matrix ligands or to cause metastasis[11] and variant isoforms, with longer extracellular tails, have altered ability to complex.[12] We found that the CD44v7-10 isoform makes PC cells preferentially bind to fibronectin rather than hyaluronan; re-expression of CD44s causes the predominant ligand to revert from fibronectin back to hyaluronan.[4] In mouse xenografts of PC-3 prostate tumor, forced expression of CD44s reduced growth *in vitro* and tumorigenicity,[5] and our use of RNAi against CD44v7-10 in xenografts yielded similar effects (unpublished results).

In PC, calcitonin (CT) acts as a paracrine growth factor that up-regulates CD44 variant.[4,6] In histologic specimens PC, but not benign secretory epithelium, contains CT[13] and its receptor (CTR),[14] and CT exerts paracrine effects that promote proliferation,[15] invasion,[16] and metastasis.[17] CTR, essential for prostate cancer tumorigenicity,[18] is coupled to the transduction protein, $G_s\alpha$. We have shown that CT promotes alternate splicing leading to CD44v7-10 mRNA and protein[4,6] by acting through $G_s\alpha$ signaling.[3] $G_s\alpha$ stimulates the cyclic AMP signaling cascade[17,19] and protein kinase A (PKA).[16]

PKA, in turn, acts on the 3 main MAPK pathways: a growth factor-responsive pathway that uses MAP2K (called MEK) as key downstream effector; and two stress-activated pathways, c-jun N-terminal kinase (JNK), and p38 kinase, that respond to stress including cytokines, osmotic shock, and irradiation. CD44v activate MAPK pathways,[20] sometimes by functioning as co-receptors for growth factors.[21] MAPK pathways, in turn, can cause CD44 alternative splicing to include variant exons.[22] Oncogenes such as *ras*[7,23] and mitogens using the MEK-ERK, MAP kinase (MAPK) pathway[7] but not the p38 pathway[24] induce CD44 promoter activity and increase expression of certain CD44v. To test whether these influences modulate RNA levels and alternative splicing of CD44 in PC, we studied the CT signaling system, PKA, and MAPK pathways. CD44 RNA and protein levels were measured.

## Material and Methods

*Cell lines*

PC-3 cells (American Type Culture Collection, Manassas, VA) were incubated in F12-K medium, 10% fetal calf serum, and antibiotics at 37ºC in a 5% $CO_2$ incubator. $G_s\alpha$-QL cells, CT-, and CTR- cells were gifts of Dr. Girish Shah, Univ. of Louisiana-Monroe.[17] The $G_s\alpha$-QL cells were derived from metastasizing PC-3M cells stably transfected by a plasmid that directs expression of mutant, constitutively active $G_s\alpha$.[17,19] These three cell lines were grown in RPMI

1640 with L-glutamine, 5% fetal calf serum, 15% horse serum and antibiotics. Benign BPH-1 cells (from Dr. Simon Hayward, Vanderbilt Univ., Nashville, TN) were grown in RPMI with 10% fetal calf serum and antibiotics. For each experiment, cells in a flask were trypsinized and saline washed to remove trypsin. 200,000 cells were plated per well on a 6-well plate. Cells were adherent and 80% confluent for all experiments. Each treatment was applied to three wells, with three other wells as mock treated controls.

*Effect of exogenous calcitonin*

PC-3M cells express both CT and CT-receptor (CTR).[16] To test the effect of solely exogenous CT on CD44, we used the derivative, CT-minus (CT-). CT- cells have endogenous CT stably knocked down to undetectable levels using anti-CT hammerhead ribozymes.[25] Salmon CT (BAChem, Torrance, CA) was used at physiologic 50 nM dose[14,16], which effectively alters CD44,[6] or at 250 nM. To detect acute versus long-acting effects on RNA and protein levels, cells were treated with 50 nM CT harvested after 3 h, or 48 h. To determine whether CT effects on CD44 proteins resulted from *de novo* protein synthesis versus protein stabilization, cycloheximide (10 nM) was given to CT- cells after 3 hr of CT exposure, and cells harvested 1 hr, 3 hr, 6 hr, and 9 hr subsequently, similar to a prior CD44 study.[26] Also tested were highly invasive, CT positive $G_s\alpha$-QL cells.[17] Finally, to rule out non-CTR-mediated CT effects, two negative controls were tested: PC-3 cells (shown to be negative for CT receptor[14]), and cells called CTR-, derived from PC-3M cells after anti-CT receptor ribozyme knockdown of CTR.[18] CTR- cells have very low levels of CD44v protein.[4]

*Inhibition of protein kinase A and MAPK components*

$G_s\alpha$-QL cells were chosen for these studies because they have the highest baseline CD44v.[3] Protein kinase A inhibitor H-89 (in 50% ethanol) (Calbiochem, La Jolla, CA) was added to cells in fresh medium (1 mL/well) at 1 µM, previously shown effective in nerve cells[23] or at a 10 µM dose. Cells were incubated with H-89 for 24 hours[27] then harvested.

In similar assays, either 10 µl of 1 mM JNK inhibitor (SP600125, Calbiochem) or 12.5 µl of 2 mM MEK inhibitor (PD98059, Calbiochem) in water were added, yielding concentrations previously shown effective: 10 µM for JNK[28] and 25 µM for MEK (personal communication, Dr. Bolin Liu). p38 kinase inhibitor (SB203580, Calbiochem) was used at 10 µM[29,30] in DMSO, and control cells received DMSO only. Cells were incubated for the optimum time of 48 h[28] to show effects.

*Interaction of CT with MAPK pathways*

Based on results above, we tested the effect of pretreatment with MEK or p38 kinase inhibitors on CT-mediated alteration of CD44 expression. 25 µM MEK inhibitor or 10 µM p38 kinase inhibitor was added to CT- cells 4 hours prior to administering 50 nM CT. Cells were harvested after 48 h as above.

*Real time TaqMan RNA analysis*

Total RNA was prepared from cell pellets using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNAse-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 µg total RNA in 20 µl reaction mixture as we did previously.[9] At least triplicate samples were run using a primer/probe set for all CD44v that brackets the entire variant region,[6] one for CD44 total that binds a standard exon, and 18S ribosomal RNA. Quantitative PCR reactions were optimized to 4 µg cDNA (0.16 µg with 18S) plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 µl. The amplification protocol was as follows: hold 50ºC 2 min, 95ºC 10 min, then 40 cycles of (95ºC for 0:15 and 60ºC for 1:00) using the ABI Prism 7700 cycler (Perkin-Elmer, Waltham, MA). Primer/probe sets for CD44v were: forward, AACGCTTCAGCCTACTGCAAA; reverse, TCTTCCAAGCCTTCATGTGATG; probe, GATTTGGACAGGACAGGACCTCTTTCAATG. For CD44 total we used forward,

CAACTCCATCTGTGCAGCAAA; reverse, GTAACCTCCTGAAGTGCTGCTC; probe, CATATTGCTTCAATGCTTCAGCTCCACCTG. Primer and probe sets for 18S were proprietary to the manufacturer.

*Western blot analysis*

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) with protease inhibitor Complete-mini tablet (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. Samples were resolved on SDS-PAGE using 25 µg sample/lane with the NuPAGE system (Invitrogen, Carlsbad, CA). 5 µl of Rainbow protein marker (RPN 756, Amersham, Piscataway, NJ) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used. To assess CD44v9 (the largest component of the overexpressed CD44v7-10) the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 standard was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA, 1:2000), which binds all forms of CD44. Anti-β-actin antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 20mM Tris pH 7.5 and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) was added at 1:9000 dilution in 5% skim milk for 1 hr. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

*Statistical analysis*

TaqMan data were analyzed by the $2(-\Delta\Delta C_T)$ method[28] to determine fold change in gene expression (mock treated cells=1.00). The $\Delta C_T$ was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA $C_T$s. The $\Delta\Delta C_T$ was obtained using the mean $\Delta C_T$ of mock treated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$.

## Results

*Calcitonin increases CD44*

In the PC-3M-derived CT- cells, a 50 or 250 µM CT dose after 48 h had little effect on the total amount of CD44 RNA, but the CD44v was tripled (**Fig. 1a**). Although different binding affinities of primer/probe sets preclude determining CD44v as a percent of CD44 total, the *relative* percent of CD44v RNA can be calculated by the $2(-\Delta\Delta C_T)$ method, as increasing fivefold after 50 µM CT. The same response, but less marked, was seen in $G_s\alpha$-QL cells, at 50 and 250 µM doses. In CTR- cells and PC-3 cells—both lacking CTR—exogenous CT had little effect. Similarly, BPH-1 cells responded to CT with very slight stimulatory effect on CD44v, and no effect on CD44 total. At the protein level, however, the CT- cells treated with CT showed increases in both total and variant CD44 after just 3 h (**Fig. 1b**) and at 48 h (**Fig. 1c**). The stimulation of CD44v protein was not attenuated by cycloheximide up to 9 h after CT (data not shown) suggesting that *de novo* protein synthesis is not required.

*Protein kinase A and MAP kinase pathways and their interaction with calcitonin*

$G_s\alpha$-QL cells have high basal levels of CD44v7-10; for this reason, these cells were used to examine the effects of protein kinase A (PKA) and MAPK pathway inhibitors. PKA inhibitor lowered CD44 total and CD44v mRNA (**Fig. 2a**) and dose-dependently decreased protein for both (**Fig. 2b**). Downstream to PKA, inhibition of MEK significantly decreased CD44 total ($p=0.001$) and non-significantly decreased CD44v. In contrast, inhibition of JNK had no significant effects. p38 inhibitor led to a larger, significant decrease in CD44 variant and a smaller significant decrease in CD44 total (**Fig. 2a**). MEK and JNK inhibitors were also tested in PC-3 cells and had no effect (data not shown). MEK inhibitor was also tested in BPH-1 cells, in which it reduced CD44 total and variant RNA.

To examine the dependence of CT effects on MAPK pathways, the CT- cells were pretreated with p38 inhibitor 4 h prior to administration of CT. Results were similar to p38 inhibitor alone: more than 50% decrease in CD44 total but none in CD44v (**Fig. 3**). This lack of CD44v suppression contrasts with p38 inhibitors marked CD44v suppression in $G_s\alpha$-QL cells (**Fig. 2a**), which have far higher CD44v.[4] This suggests that CT mediated splicing is through p38 kinase. In further support of this, the expected CT induced tripling of CD44v mRNA in CT- cells (**Fig. 1a**) was prevented by p38 inhibitor pretreatment. Pretreatment with MEK inhibitor before CT also blunted the expected rise in CD44 variant mRNA seen in **Fig. 1a**, and JNK inhibitor pretreatment had no effect (data not shown).

## Discussion

Here, we demonstrate that calcitonin (CT) causes CT receptor-dependent increases in CD44 alternate splicing in prostate cancer (PC), apparently mediated through p38 kinase. Furthermore, transcription but not splicing appears to require the MEK/ERK (MAPK) pathway. Proposed interactions are shown (**Fig. 4**).

Paracrine CT is among several growth factors that interact with CD44.[22] In our prior *in vitro* studies up to 100 nM exogenous CT[6], or CT originating endogenously (in a PC-3 derivative called CT+ [4]), increased CD44v7-10 expression at the mRNA and protein levels. This was also observed in LnCaP, PC-3, and PC-3M derived cells; however, we had not examined total CD44 previously. Here, we used CT-minus (CT-) cells, an androgen-independent PC-3M derivative, to exclude all endogenous CT influence, so any effects would be attributable solely to exogenous CT. Since the $K_d$ of CTR is 4-21 nM,[15] the 50 nM dose we used should saturate it. In CT- cells, the aberrant splice product is CD44v7-10.[6] This action occurred also in $G_s\alpha$-QL cells, which are CTR+. Supporting the view that this stimulation was CT-receptor mediated and not nonspecific,

administering CT to CT-receptor negative PC-3 or CTR- cells did not have this effect. In further support of this interpretation, with benign BPH-1 cells, which are also negative for CT and CTR (personal communication, GV Shah), exogenous CT exerted no effect.

It is a novel observation that CT increases CD44v mRNA and protein as early as 3 h in cells that are CTR+, and has little effect on CD44 total. A response to CT should occur in the first several hours (personal communication, GV Shah), and indeed these increases were evident in CD44 protein levels at 3h and 48 h. The stimulation of CD44v at 250 nM CT is unlike the effect on invasion, with dose-dependent inhibition at concentrations exceeding 50 nM.[16] The time-dependent reduction in protein after cycloheximide shows that the effect on CD44 variant protein is not the result of protein stabilization but reflects de novo protein synthesis from RNA.

Since the MEK/ERK pathway and the two stress-activated MAPK pathways are implicated in androgen-independent prostate cancer growth,[32] we tested all three for modulation of CD44. We had found that CD44v7-10 protein was overexpressed in xenografts of $G_s\alpha$-QL compared to PC-3M[3]; and i*n vitro*, pharmacologic stimulation of $G_s\alpha$ or adenylyl cyclase raised CD44v7-10.[6] Using a PKA inhibitor, we found reduced total and CD44v7-10 mRNA, suggesting involvement of MEK pathway. PKA acts through protein tyrosine phosphatase (PTP) on MAP kinase kinase kinase (MAP3K) which activates to MAP2K (called MEK). MEK in turn activates extracellular signal-regulated kinases (ERK). Over half of PC cases have activated MEK-ERK signaling, shown by immunohistochemistry for p44/ERK1 and p42/ERK2.[33]

To assess MAPK inhibitor effects, we chose $G_s\alpha$-QL cells, derived from metastasizing PC-3M cells that stably express *gsp* mutant, constitutively active $G_s\alpha$[17,19] because they have high baseline CD44v7-10.[3, 6] We found MEK inhibitor caused similar percent decreases in CD44 total and CD44 variant, implicating MEK in transcription if not CD44v splicing. Similar effects have been noted with ras oncoprotein, which acts on the MEK-ERK pathway.[24] Ras activation

can induce CD44 promoter activity in fibroblasts, as shown using transient cotransfection of c-ras expression constructs and CD44 promoter reporter gene constructs.[7] Leakiness of splice control is proposed to lead to increased CD44v.[7] In activated T-lymphocytes during the immune response, mutant *ras* stimulation of MEK-ERK pathway increases CD44 total mRNA and triggers inclusion of CD44v exons in the mature RNA[24]; from our experiments, MEK seems to be active at least in CD44 transcription in $G_s\alpha$-QL cells.

Some studies have suggested a positive feedback loop coupling MEK/ERK pathway and CD44v splicing. Activation of *ras* oncogene in rat fibroblasts[7] and HeLa cervical cancer[23] and of its effector, the MAP kinase pathway in T-cells[24] both upregulate CD44v splicing. CD44 variants, in turn, serve as coreceptors for growth factor receptors that activate ras,[23] or form complexes with receptor tyrosine kinases such as c-met[8,21] to mediate cell signaling. Moreover, CD44v6 promotes T-cell proliferation by persistently activating MAP kinases,[20] and CD44v8-10 causes apoptosis resistance in small cell lung cancer by activating Rho-stimulated focal adhesion kinase (FAK).[34]

We examined p38 kinase in benign and PC cells. In BPH-1, CD44 total RNA decreased with p38 kinase inhibition but variant form was unchanged. Since benign prostate lacks the aberrant splicing leading to CD44v7-10[3], but CD44v3-10 expression is present,[35] the latter may be the form detected in benign cells. In $G_s\alpha$-QL cells, a more marked effect on total and variant CD44 was seen. However, p38 may have CT-independent actions. Similar to our current and previous[6] findings with CD44 and $G_s\alpha$, p38 and MEK (but not JNK) were responsive to G-protein-coupled P2Y purinoceptor agonist ATP in PC-3 cells (CTR-negative), and these 2 pathways were required for invasion.[30] p38 has recently been recognized as a cell proliferation and survival factor in PC,[36] partly by regulating IL-6 secretion.[32] Taken together with our findings about the MEK/ERK role in CD44 transcription, this could reflect convergence of the

ERK1/2 and p38 systems in activating the MNK1 kinase, which enhances transcription of certain targets,[37] suggesting a common final pathway that stimulates CD44 expression in PC.

We tested possible JNK pathway effects on CD44, not previously examined in the literature. JNK appears mainly important in PC apoptosis[29,38] and promoting chemotherapy susceptibility *in vitro* and in xenograft tumors of cancers such as non-small cell lung cancer.[28] Recent evidence suggests that CT receptor effects are mediated by the urokinase type plasminogen (uPA) pathway in prostate cancer tumorigenicity[18] and uPA, acting through focal adhesion kinase and Rho protein, affects the JNK pathway. JNK inhibitor slightly decreased CD44 total protein and did not change CD44v mRNA or protein in $G_s\alpha$-QL cells. Our data suggest that JNK is a minor influence on CD44 expression.

Inhibition of p38 and MEK pathways affected CD44 in $G_s\alpha$-QL cells. To investigate whether either might mediate CT's effects on CD44, we administered CT to CT- cells after blocking either one of these pathways. CT- cells have low baseline CD44v,[6] and p38 inhibitor did not suppress the CD44v, but it blocked the expected stimulation of CD44v by CT, suggesting that p38 mediates CT-stimulated alternative splicing. The marked CD44v suppression seen in $G_s\alpha$-QL cells, which have endogenous CT and high baseline CD44v, adds support for this interpretation. It is tempting to speculate that CT signaling, raising cAMP, may act through the effector, "exchange factor directly activated by cAMP" (Epac, **Fig. 4**). Epac has been shown to activate p38 kinase and mobilize intracellular calcium in neurons.[39] This PKA-independent mechanism would explain why PKA affected primarily CD44 transcription, yet p38 showed evidence of an additional effect on splicing. MEK inhibitor alone reduced CD44 total and variant in the PC-3M family of cells; however, MEK blockade prior to CT only slightly blunted this effect.

To our knowledge, this is the first report of how interactions in PC between CT, and MAP kinase pathways, dysregulate the expression and splicing of the CD44 molecule. CD44 variant isoforms, probably through altered multimerization[12] and ligand binding,[4] allow prostate cancer invasion.[3, 6] This knowledge may find application in targeting the aberrant splicing of CD44 in PC by gene therapy, molecular inhibitor therapy, or for sensitization to radiotherapy.

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## Figure Legends

**Fig. 1a**. RNA response to exogenous CT at 48 h is calcitonin (CT) receptor-dependent. CD44 total and variant RNA are quantified by triplicate TaqMan RT-PCR experiments. In benign BPH-1 cells, no response is observed to exogenous calcitonin (CT). CT- cells, a PC-3M derivative that lack endogenous CT but have CT receptor, showed a decrease in CD44 total RNA but an increase in variant RNA. The $G_s\alpha$-QL cell derivative showed the same trend. In CT-receptor-negative CTR- and PC-3 cells, there is no significant response to CT. Error bars are standard deviation. *p=0.01; † p=0.05 with respect to mock treated controls.

**Fig. 1b**. Western blot analysis. Exogenous calcitonin given to the CT- cells for 3 h stimulates expression of CD44s at 75 kD (top), and CD44v at 180 kD and its cleavage products below 97 kD. Comparison is shown to cells with no treatment (NT). β-actin analysis control confirms equal protein loading (bottom).

**Fig. 1c**. This stimulatory effect persists at 48 h and at doses of 50 mM or 250 mM exogenous calcitonin.

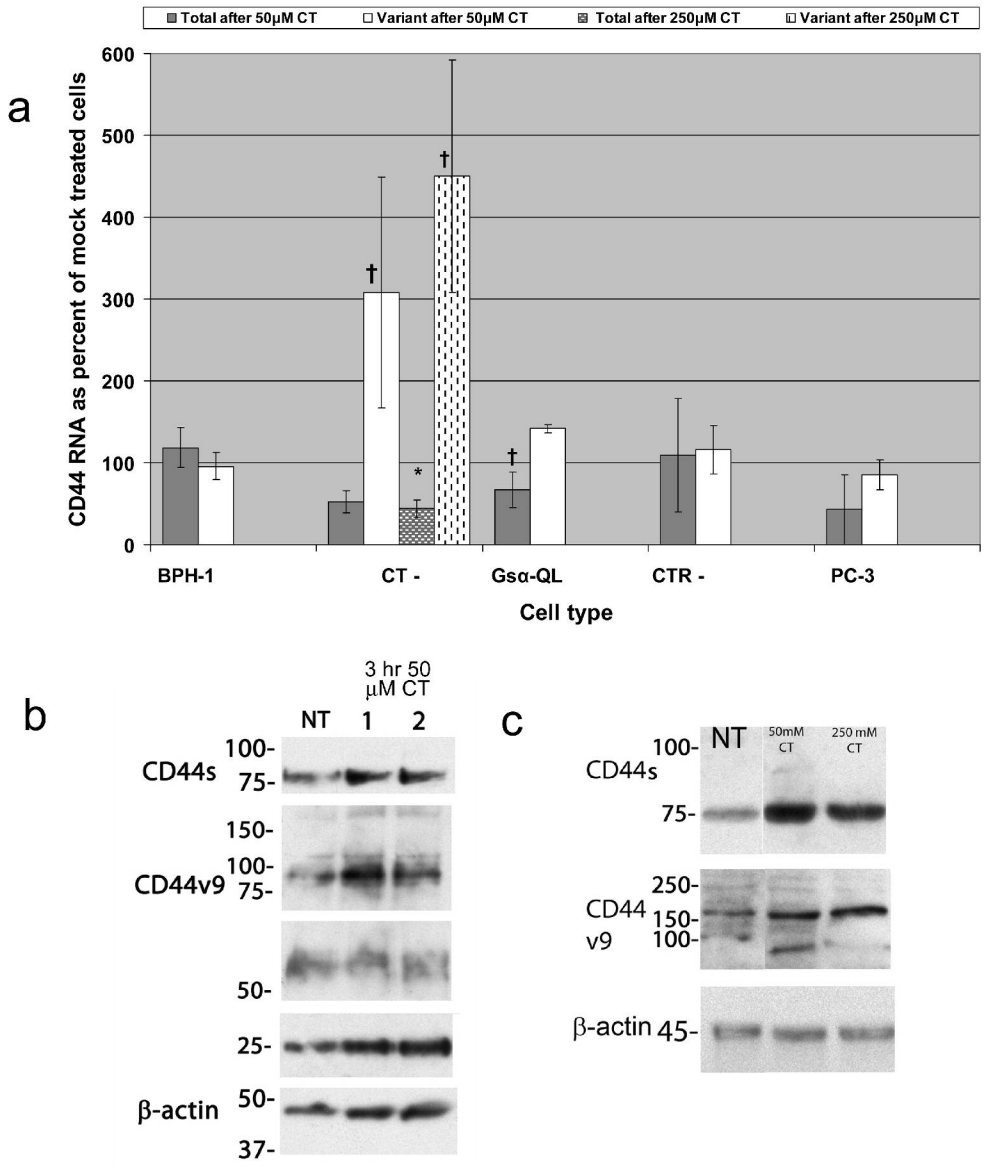
**Fig. 2a**. RNA response to molecular inhibitors. CD44 total and variant RNA in triplicate TaqMan RT-PCR experiments. In subconfluent $G_s\alpha$-QL prostate cancer cells, significant decreases of about 50% or more were observed after protein kinase A (PKA) inhibitor H-89, or inhibitors of downstream signaling pathways MEK or p38 kinase, but not JNK inhibitor. Error bars are standard deviation. *p=0.01; **p=0.001; †p=0.0002; ***p=0.0001 with respect to mock treated controls.

**Fig. 2b**. Western blot analysis. Protein kinase A inhibitor exerts a dose-dependent decrease on CD44s and CD44v compared with EtOH control in $G_s\alpha$-QL cells.

**Fig. 3.** Blockage of p38 kinase in CT- cells counteracts exogenous calcitonin (CT) stimulatory effect on CD44 variant RNA expression in CT- cells. Triplicate TaqMan RT-PCR experiments. While p38 blockade does not affect the CT-induced decrease in CD44 total, it does abrogate the expected tripling (Fig. 1) of CD44 variant. Conversely, CT did not significantly counteract MEK inhibition's effects on decreased total and variant CD44. *p=0.02; **p=0.006 with respect to mock treated controls.

**Fig. 4**. Proposed effects of calcitonin and MAP kinase pathways on CD44 expression in androgen-independent prostate cancer.

Calcitonin (CT) binds to its receptor (CTR), which is coupled to the $G_s\alpha$ transduction protein. $G_s\alpha$ activity, mediated through cAMP, activates protein kinase A (PKA) [41]. PKA activates the MAPK kinase (MEK)-extracellular regulated kinase (ERK) pathway, that facilitates CD44 transcription. CT also induces splicing of CD44 to include v7-10, dependent on p38 but not on PKA. p38 may be induced by Exchange protein activated by cAMP (Epac). p38 could affect splicing machinery directly, through other downstream effectors, or by causing release of intracellular $Ca^{2+}$.

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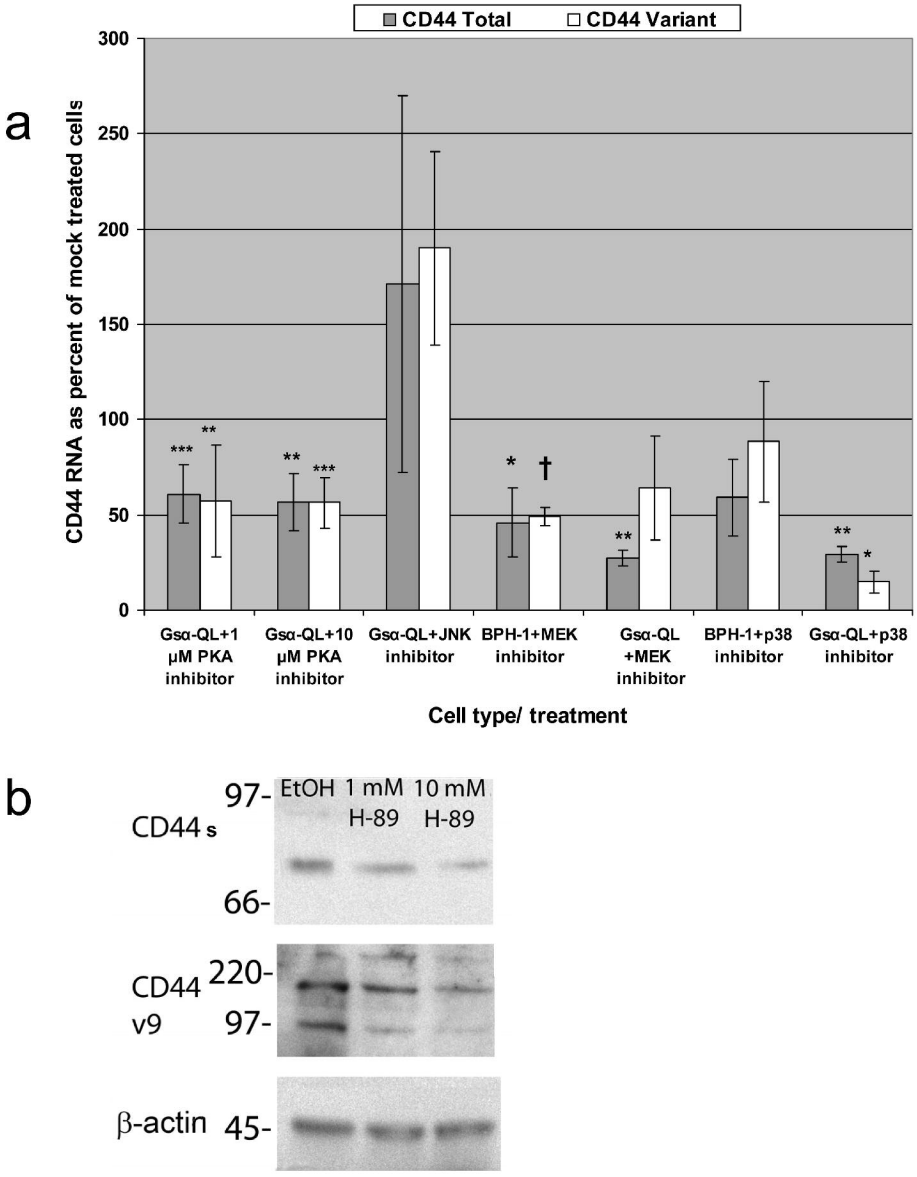


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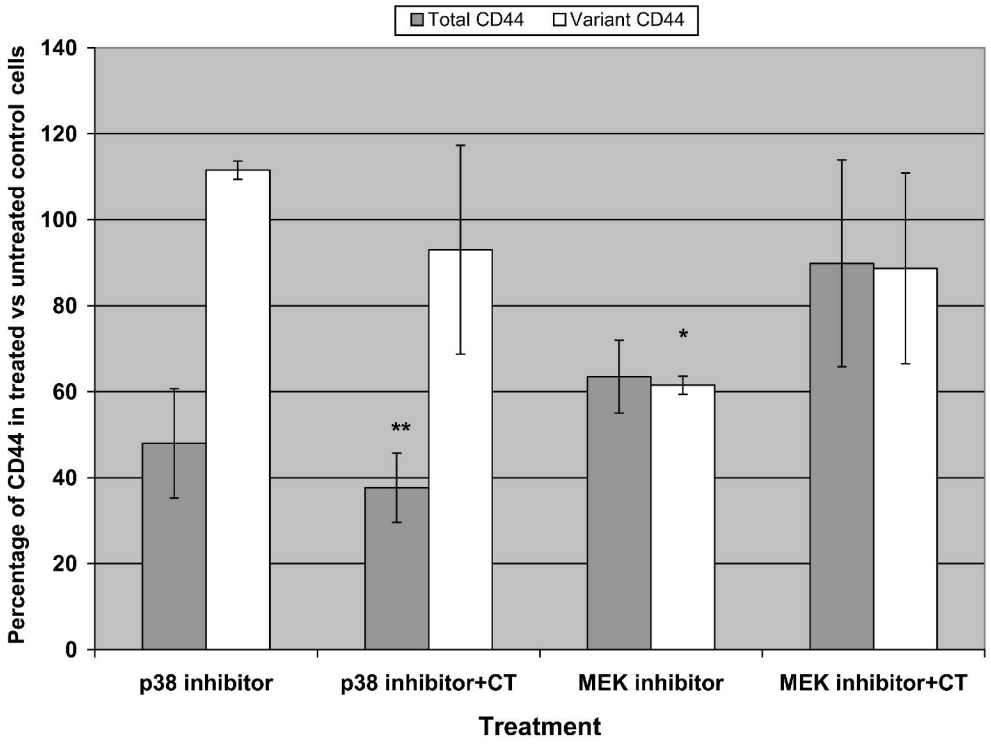


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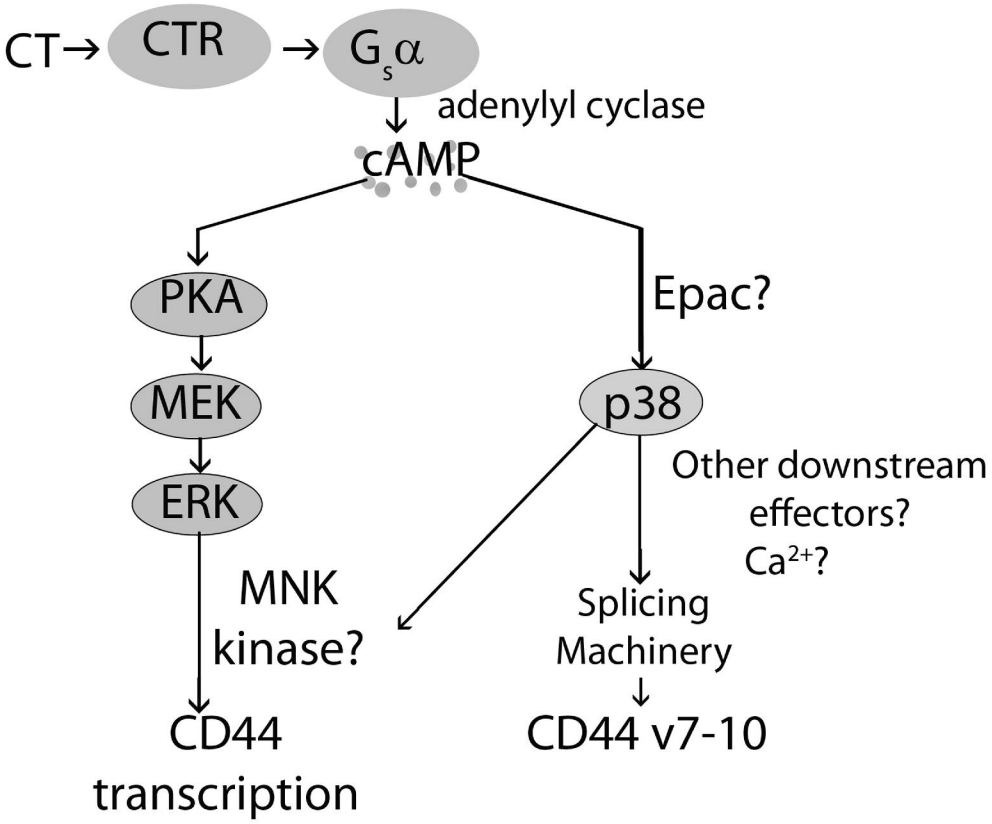


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